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14. ABSTRACT We have made substantial progress in obtaining skin biopsies with subsequent fibroblast cultures of a number of sporadic ALS, familial ALS, and control subjects in the last year. We have created numerous iPS cell lines from these patient samples and have characterized their long-term differentiation into astroglia. We now have data from the in vivo transplantation of these astroglial progenitors into rat spinal cords. We have characterized several control and 1 ALS cell line with regard to their survival, differentiation, migration, and phenotypic effects on the host animals. Our data demonstrate that there are differences amongst the cell lines with regard to these properties but we have not appreciated obvious differences with regard to ALS cells when compared with control astroglial cells. Finally, we have demonstrated, in preparation for our therapeutic approaches, that these glial progenitor cells continue to mature into astrocytes following transplantation.					
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INTRODUCTION:

The overall objective is to examine whether human iPSC-GRPs (glial restricted precursors) derived from either sporadic ALS, familial (SOD1-mediated) ALS, or control subjects have the same capacity for engraftment, survival, and neuroprotective qualities following transplantation. It is not known whether iPSC-GRPs from ALS patients will in fact be normal (and thus possibly neuroprotective) or whether these iPSC-derived cells may in fact harbor ALS-specific abnormalities which may lack benefit or, potentially exacerbate disease. By comparing normal iPSC-GRPs with sALS iPSC-GRPs and fALS iPSC-GRPs we will also learn about inherent differences in astrocyte biology related to ALS which will provide potential insights into disease mechanisms.

BODY:

Aim #1. Generation of human induced pluripotent stem cell-derived glial restricted precursors (iPSC-GRPs) from ALS subject fibroblasts

Task 1. Skin biopsy of subjects with ALS to obtain fibroblasts (Months 1-18)

Total of approximately 111 subjects biopsied to date). These include subjects with familial ALS (these include SOD1, ANG, FIG4, FUS, and now C9ORF72 mutations), as well as subjects with sporadic ALS and control subjects (**Table 1**).

Table 1

KNOWN MUTATIONS	FAMILIAL	FIBROBLASTS	iPS
SOD1			
N139K	1	x	1
A4V	6	x	4
D90A	2	x	2
V148G	1	x	1
I113T	4	x	1
I112T	1	x	1
L144P	1	x	1
C38G	1	x	1
D91A	1	x	1
E49K	1	x	1
E100G	1	x	1
G86R	1	x	1
FIG 4	1	x	1
FUS	3	x	3
TDP43	2		2
C9ORF72	1	x	1
ANG	1	x	1
TOTAL	29		

OTHER		
SPORADIC		iPS lines
SLOW PROGRESSING > 5 YRS	9	7
TYPICAL PROGRESSION < 5 YRS	38	4
ALS/FTD	3	1
UNKNOWN FAMILIAL	4	1
PLS	7	2
LMN ONLY	5	1
UMN ONLY	1	
C9ORF72	4	2
PSEUDO BULBAR	1	
KENNEDY'S	2	
CONTROLS	5	3
RELATED CONTROLS	3	
TOTAL	82	

iPS lines completed	
fALS	27
sporadic ALS	12
other	3
controls	7

Task 2. Culture of human fibroblasts (Months 1-24)

Total of approximately 111 fibroblast lines cultured to date. Many of these lines have already been frozen down to allow for future use. (**Table 1**)

Task 3. Generation of human iPSC from ALS and control subjects (Months 1-24)

In collaboration with Dr. Song as well as a collaborative effort with Dr. Jeffrey Rothstein at Johns Hopkins and the Johns Hopkins Institute of Cell Engineering Stem Cell Core, we have now generated 27 FALS iPSC lines, 12 sporadic ALS iPSC lines and 7 control iPSC lines (**Table 1**).

Task 4. Characterization of iPS cells (Months 1-24)

iPSC have been generated from patients and we have verified that ALS and control iPSCs maintain their pluripotency and have normal karyotypes. All cell lines listed in **Table 1** have a normal karyotype.

Task 5. Differentiation and characterization of iPS cells from ALS subjects into glial restricted precursors and astrocytes (Months 1-24).

We have now generated multiple lines of iPSC-derived astrocytes using the protocol outlined in **Figure 1**. iPSC are maintained as we have previously described and then undergo a process of neural induction followed by approximately 60 or more days of maturation into astrocytes using 10% fetal bovine serum (FBS). We have now initiated an in vitro evaluation of iPSC-derived astrocytes to ascertain whether they express appropriate astrocytic markers including GFAP, the astrocyte-specific glutamate transporter GLT1, connexin 43, aquaporin 4, the cell surface marker CD44 (a marker of astrocyte precursor identity), and the intermediate filament vimentin. Our data do not indicate differences in the capacity for differentiation between ALS and control astrocytes (**Fig. 2**).

Figure 1. Protocol for differentiation of iPSC into human iPSC-derived astrocytes

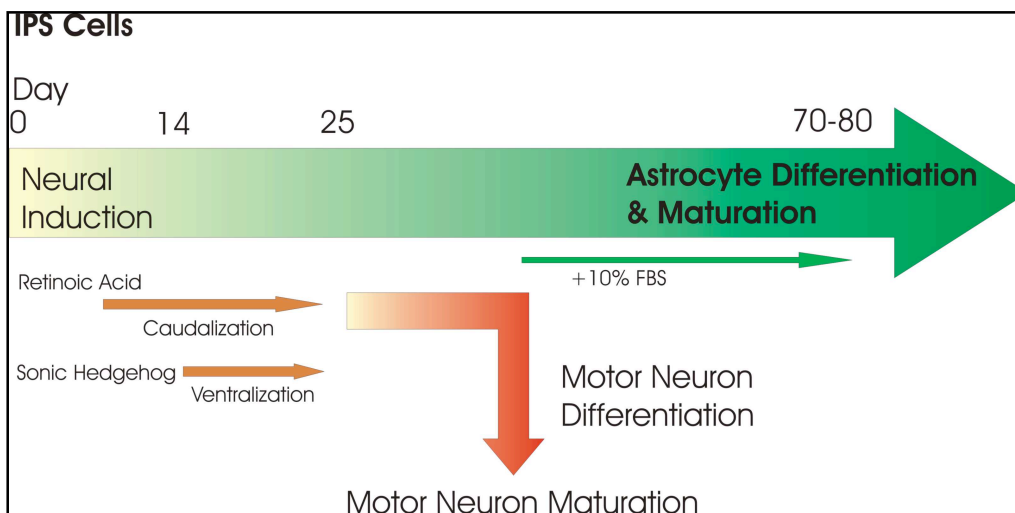
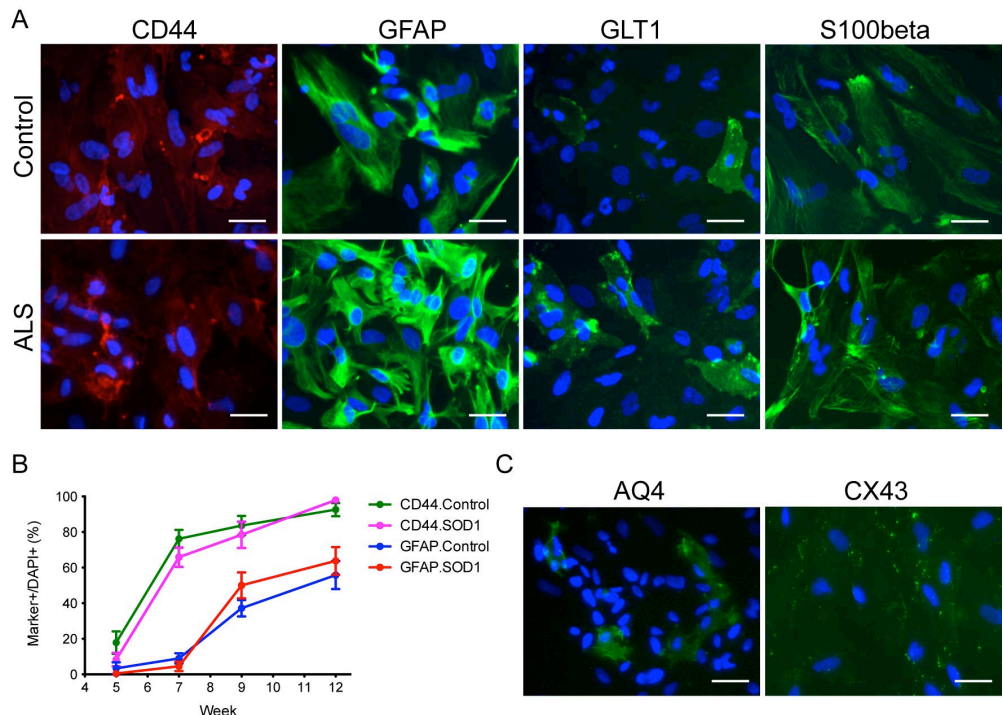


Figure 2. iPSC-derived astrocytes from both control and SOD1^{A4V} ALS patients express astrocyte-specific proteins.

(A) Expression of CD44 (red), GFAP (green), GLT1 (green) and S100b (green) by control (line 006) and fALS-iPSC (line 002 SOD1^{A4V}) derived astrocytes at week 13-14.

(B) Dynamic examination of CD44 and GFAP expression by control and A4V-astrocytes.

(C) Expression of aquaporin 4 and connexin by a fALS-astrocyte (line 001 SOD1^{N139K}). Nuclei were stained with DAPI (blue). Size bar, 20µm.



Aim #2. In vivo comparison of iPS cell-derived glial restricted precursors (iPSC-GRPs) from control, sporadic ALS, and familial ALS (SOD1) following transplantation into wildtype spinal cord

Task 1. Transplantation of iPSC -GRPs into wildtype rats

a. Characterization of iPSC -GRPs cell survival, differentiation (Months 1-24)

Transplantation of iPSC-derived astrocytes to the rat spinal cord

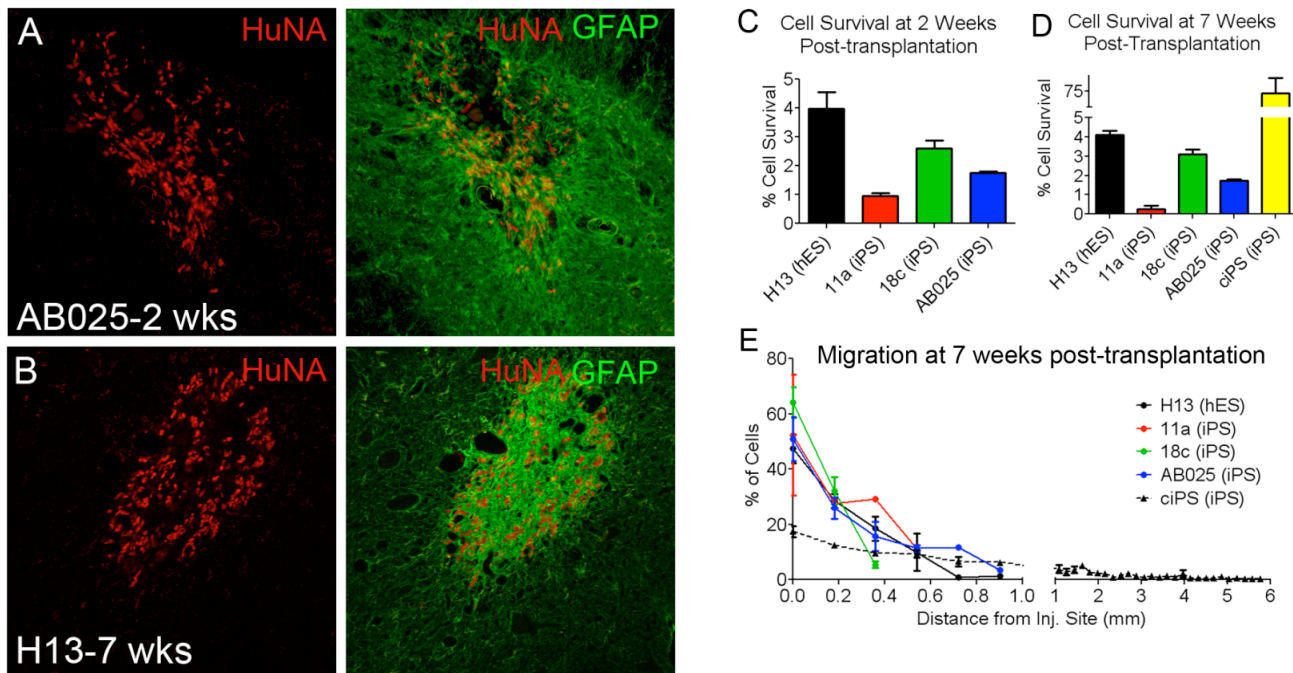
To evaluate the iPSC-derived GRPs propensity for engraftment, the cells were transplanted to the ventral horn of the rat cervical spinal cord using the transplantation paradigm previously described by our lab. Each rat received one or two bilateral injections at level C5 of 150,000 cells per injection. Prior to the injection and for the remainder of the study, rats were given high-dose cyclosporine to prevent immune rejection of the grafted cells. Rats were sacrificed at 2, 7, or 12 weeks post-transplantation (**Table 2**).

Table 2

Cell Line	Cell Type	Source	Age/Sex	In Vitro Diff. (Days)	Weeks until sacrifice (n)		
					2 wks	7 wks	12 wks
H13	hESC	Columbia	0/M	113	2	2	
11a	hiPSC	Columbia	36/M	112	2	3	
18c	hiPSC	Columbia	48/F	115	2	3	
AB025 (ALS--FUS)	hiPSC	MGH	50/F	114	1	3	
ciPS	hiPSC	Iperian	10/M	95		3	3

At all time points, the transplanted cells could be localized in the grey matter of the spinal cord by staining for human-specific nuclear antigen (HuNA) (**Fig. 3 A and B**). For 3 of the 4 iPSC lines as well as the hESC line, survival at 2 and 7 weeks was low (<5%) (**Fig. 3C and D**) and the transplanted cells mainly resided near the site of injection with migration of less than 1 mm (**Fig. 3E**).

Figure 3



In contrast, transplantation of astrocytes derived from the ciPS cell line resulted in much improved survival (75% at 7 weeks) compared to other lines with impressive migration of up to 6 mm from the site of injection (**Fig. 3**). At 12 weeks post-transplantation of the ciPS cells, cell survival was quantified at 275%, indicating the ciPS cells were continuing to proliferate and migrate (**Fig. 3**). Indeed, staining for the proliferation marker Ki67 revealed that 19% of the HuNA+ cells were proliferating at 7 weeks post-transplantation (not shown). By 12 weeks, many of the HuNA+ cells stopped dividing with only 6% expressing Ki67. In rats receiving ciPS astrocyte transplants, HuNA+ cells could be localized after 12 weeks throughout the entire grey and white matter of the spinal cord (not shown). The HuNA+ ciPS cells were distributed evenly throughout the spinal cord instead of clustered at the injection site and in some sections, comprised over 50% of the DAPI+ cells in the spinal cord (**Fig. 4A and B**).

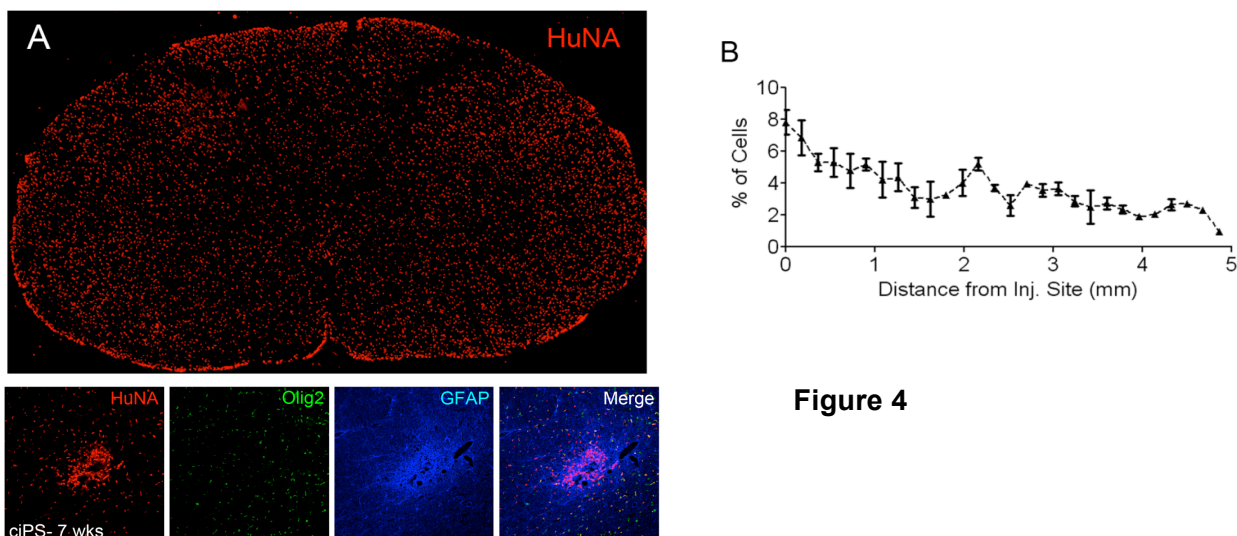
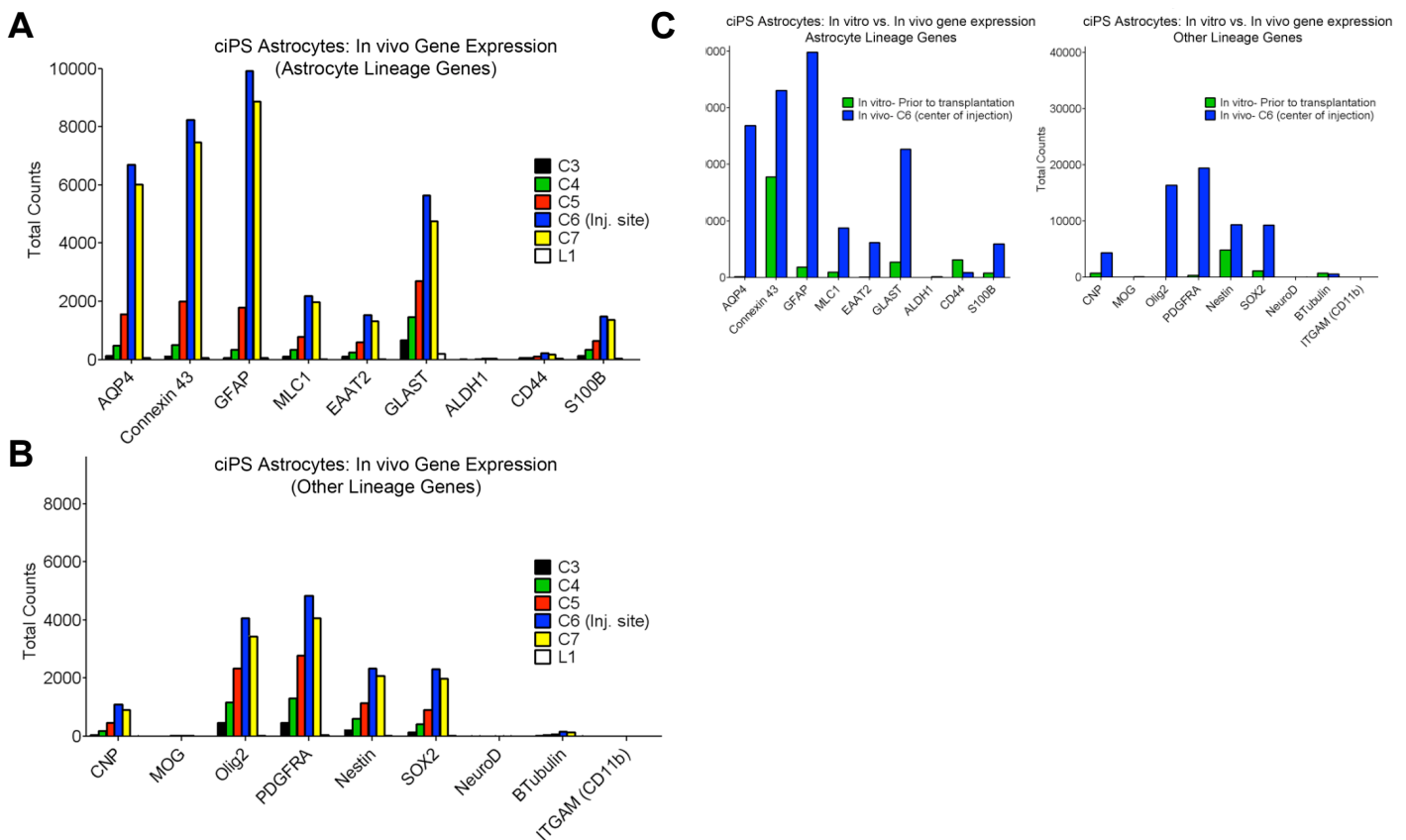


Figure 4

hiPSC continue to mature in vivo (c-iPSC)

Using Nanostring human specific primers to investigate how GRPs mature following transplantation into the spinal cord, we observed that c-iPSC-derived GRPs expressed astrocyte (**Fig. 5A**) lineage genes most abundantly at levels C6 and C7 of the spinal cord. This corresponds to the region of transplantation and provides us with the ability to track these cells in vivo and to establish that in fact we have appropriate astrocytic markers of these cells in vivo. Because these are glial progenitors, we also detected human specific oligodendrocyte genes in those same transplanted regions (**Fig. 5B**), although the levels of gene transcripts were not as robust as the astrocyte gene transcripts. In addition to establishing that the c-iPSC-derived GRPs could survive and express astrocyte and oligodendroglial genes, we also wanted to know if there was further maturation of these cells following transplantation. In Fig. 5C the **in vitro** levels of astrocyte and oligodendrocyte transcripts are represented by green bars. Following transplantation into rats and sacrifice 12 weeks after transplantation, we saw a dramatic increase in astrocyte and oligodendrocyte gene transcripts **in vivo** (blue bars). These data suggest that these c-iPSC-derived GRPs mature into astrocyte and oligodendrocytes. In addition, we also did not detect neuron specific gene transcripts (Neuro D or beta tubulin) nor did we observe any microglial markers (ITGAM—CD11b). These data suggest that the iPSC-derived GRPs maintain their fidelity to a glial lineage by only differentiating into astrocytes and oligodendrocytes.

Figure 5



Planned experiments Year #3

Aim #3. Determine the capacity for neuroprotection of iPSC-derived glial restricted precursors (iPSC-GRPs) following transplantation into the SOD1^{G93A} rat model of ALS.

Task 1. Transplantation of iPSC-GRPs into SOD1^{G93A} rats

Dr. Nicholas Maragakis, Johns Hopkins University

a. Behavioral assessment of (iPSC-GRP transplanted GRPs in the SOD1^{G93A} rat (Months 12-36)

1. forelimb and hindlimb grip strength
2. Survival studies
3. Electrophysiological studies

Animals: SOD1^{G93A} Sprague Dawley Rats: Approximately 40 (10 with control iPSC-GRP, 10 sALS iPSC-GRP, 10 fALS iPSC-GRPs, 10 media control injections)

b. Pathological assessment of iPSC-GRP in the SOD1^{G93A} rat (Months 12-36)

Animals: SOD1^{G93A} Sprague Dawley Rats: Approximately 40 (10 with control iPSC-GRP, 10 sALS iPSC-GRP, 10 fALS iPSC-GRPs, 10 media control injections)

KEY RESEARCH ACCOMPLISHMENTS:

--Induced Pluripotent Stem Cell lines have been created from subjects with familial ALS, Sporadic ALS, and controls

--IPS Cell-derived astrocyte progenitors have been successfully developed from subjects with familial ALS, Sporadic ALS, and controls

--Initial transplantation experiments of iPS Cell-derived glial progenitors suggest that there is variability among control iPS cell-derived lines with regard to survival, migration, and differentiation.

--Following transplantation, iPSC-derived glial progenitors continue to differentiate and develop more mature markers of astrocyte and oligodendrocyte identity.

--We have not yet appreciated any significant differences in the in vivo characterization of ALS iPSC-derived astroglial progenitors when compared with control astroglial progenitors.

REPORTABLE OUTCOMES: NONE

CONCLUSION:

We have made substantial progress in obtaining skin biopsies with subsequent fibroblast cultures of a number of sporadic ALS, familial ALS, and control subjects in the last year. We now have created numerous iPS cell lines from these patient samples and differentiated these cells into iPSC-derived glial progenitors. We have successfully, over the last year, established a paradigm for the long-term study of these cells in an in vivo environment by transplanting these cells into the spinal cords of wildtype rats. Our next year will involve the transplantation of the control iPSC-derived glial progenitors into SOD1 animal models.

REFERENCES: NONE

APPENDICES: NONE